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Tumor-induced Interleukin-10 Inhibits Type 1 Immune Responses Directed at a Tumor Antigen As Well As a Non-Tumor Antigen Present at the Tumor Site[1]

Brian K. Halak, Henry C. Maguire, Jr., and Edmund C. Lattime[2]

Immunology Program, Kimmel Cancer Center [B. K. H., E. C. L.] and Department of Medicine [H. C. M., E. C. L.], Division of Medical Oncology, Thomas Jefferson University, Philadelphia, PA 19107; and Department of Surgery, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School and The Cancer Institute of New Jersey, New Brunswick, NJ 08901 [E. C. L.]

ABSTRACT

Interleukin (IL)-10 is a potent immunosuppressive cytokine that has been found to be present at the tumor site in a wide variety of human cancers, including transitional cell carcinoma of the bladder. Using a murine bladder tumor (MB49), which we show to express the male transplantation antigen (HY), we tested the hypothesis that IL-10 at the tumor site can block the generation of a tumor-specific type 1 immune response. We show that, despite its expression of HY, MB49 fails to prime for an HY-specific type 1 (IFN-γ) response in normal female mice. Although MB49 does not constitutively produce IL-10, our data support a model whereby MB49 induces infiltrating cells to produce IL-10. This feature rendered the IL-10 knockout (KO) mouse, whose infiltrating cells are incapable of IL-10 production, a suitable model in which to study MB49 in the absence of IL-10. When injected into IL-10 KO mice, MB49 does prime for an HY-specific, type 1 immune response. Furthermore, IL-10 KO mice show prolonged survival and an increased capacity to reject tumors as compared with normal mice. We also tested the ability of tumor-induced IL-10 to inhibit immunization to a non-tumor antigen present at the tumor site. When vaccinia virus encoding β-galactosidase (β-gal) is injected into the tumors of normal mice, no β-gal-specific IFN-γ response is mounted. However, when this same viral construct is injected into the tumors of IL-10 KO mice, it produces a strong β-gal-specific, IFN-γ response. These studies demonstrate that tumor-induced IL-10 can block the generation of a tumor-specific type 1 immune response as well as subvert attempts to elicit a type 1 immune response to a non-tumor antigen at the tumor site.

INTRODUCTION

Immune responses are often categorized as either type 1 or type 2, based on the profile of cytokines produced by both $CD4^+$ and $CD8^+$ T cells (1). A type 1 cytokine profile is most often associated with cell-mediated immunity, whereas type 2 cytokines favor the development of antibody responses (2). Frequently, the effectiveness of a given immune response depends on the type of response elicited, as demonstrated by murine *Leishmania major* where a type 1 response clears the infection effectively, whereas a type 2 response fails to resolve infection (3). It is believed that cell-mediated, type 1 responses are also the most suitable for the eradication of malignant cells (4, 5). Given that type 1 and type 2 responses have been shown to cross-regulate one another, one mechanism that has been proposed as a means of immune evasion by tumors is the suppression of type 1 responses via the preferential induction of type 2 cytokines at the tumor site.

IL-10[3] is a type 2-associated cytokine that has been shown to inhibit the development of cellular immune responses via a number of mechanisms. Perhaps the best characterized of these is the ability to inhibit antigen-induced T cell proliferation indirectly by down-regulating molecules found on professional APCs, which are essential for T-cell activation (6–8). IL-10 also inhibits inflammatory cytokine synthesis by macrophages directly and can inhibit human T cell proliferation directly by blocking IL-2 production at the transcriptional level (9, 10). Furthermore, recent studies by Groux *et al.* (11) demonstrate that activation of antigen-specific T cells in the presence of IL-10 drives the differentiation of these cells toward a T-helper phenotype that is capable of blocking T-cell proliferation both *in vitro* and *in vivo*. Thus, through a variety of mechanisms, IL-10 can prevent the generation of a productive cellular immune response.

We and others have reported the presence of IL-10 at the site of progressively growing tumors in a variety of human cancers, including melanoma, non-small cell lung carcinoma, renal cell carcinoma, and bladder cancer (12–16). Moreover, increased IL-10 serum levels have been reported in patients with melanoma, renal cell carcinoma, and pancreatic, gastric, and colon adenocarcinoma (17, 18). Given the well-documented capability of IL-10 to suppress cellular immune responses, one might speculate that the observed IL-10 allows for progressive tumor growth by preventing the generation of a tumor-specific cellular immune response. However, efforts to test this hypothesis have yielded contradictory results. Studies have shown that IL-10 suppresses the tumoricidal activity of macrophages (19), inhibits cytotoxicity and cytokine production by tumor-specific T cells (20), and blocks the presentation of tumor antigens by APCs (21). These findings support the view that IL-10 plays a role in suppressing tumor-specific immune responses and thus increases tumorigenicity. In contrast, several studies using tumors genetically engineered to produce IL-10 have shown that constitutive IL-10 production at the tumor site can stimulate a tumor-specific immune response and thereby decrease tumorigenicity (22–25).

The discrepancy between these findings is intriguing and remains to a large degree unresolved. It is important to consider that the majority of studies demonstrating an immunostimulatory role for IL-10 have used tumors engineered to produce IL-10 by gene transfection, which may not mimic the natural production of IL-10 accurately in terms of concentration, timing, and location. Nevertheless, *in vivo* models directly demonstrating a role for IL-10 in the suppression of antitumor immunity remain limited. Here we describe a tumor cell line that does not produce IL-10 *in vitro* but is associated with IL-10 *in vivo*, suggesting that it induces infiltrating cells to produce IL-10. By comparing the behavior of this tumor in normal *versus* IL-10 KO mice, whose infiltrating cells are unable to produce IL-10, we provide the first *in vivo* evidence that tumor-induced IL-10 can prevent the generation of a type 1 immune response directed at a tumor-associated antigen. We report that in the absence of IL-10, animals show prolonged survival and an increased frequency of tumor rejection. Finally, we demonstrate that the immunosuppressive effects of tumor-induced IL-10 are not limited to the tumor antigen, but can also block the induction of a type 1 immune response specific for a non-tumor antigen present at the tumor site.

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[2] To whom requests for reprints should be addressed, at The Cancer Institute of New Jersey, 195 Little Albany Street, New Brunswick, NJ 08901.

[3] The abbreviations used are: IL, interleukin; APC, antigen-presenting cell; KO, knockout; pfu, plaque-forming unit(s); DC, dendritic cell; *BCG, Bacillus Calmette-Guérin.*

MATERIALS AND METHODS

Animals and Tumor. Female C57BL/6 mice, male C57BL/6 mice, and female C57BL/6-IL10tm1cgn mice (IL-10 KO) were purchased at 3–5 weeks of age from The Jackson Laboratory (Bar Harbor, ME) and maintained in our biosafety isolation animal suite for at least 1 week prior to use. The Institutional Review Board of Thomas Jefferson University approved all animal studies. The MB49 tumor cells (7,12-dimethylbenz[a]anthracene-induced in male C57BL/6 bladder epithelial cells) described originally by Summerhayes *et al.* (26), were obtained from Dr. Tim Ratliff, Washington University, St. Louis, MO, and carried *in vitro* in our laboratory.

RT-PCR. Total RNA was extracted from cells or tissues using TRIZOL Reagent (Life Technologies, Inc., Gaithersburg, MD). cDNA synthesis was performed by incubating 5 μg of total RNA in diethyl pyrocarbonate-treated water, Random Primers (Life Technologies), and reverse transcription buffer (Life Technologies) at 65°C for 10 min and then cooling the mixture immediately to 4°C. To this mixture, 10 mM DTT (Life Technologies); 2.5 mM each of dATP, dCTP, dGTP, and dTTP (Life Technologies); and 500 U of MMLV-RT (Life Technologies) were added to achieve a final sample volume of 50 μl. To complete the reverse transcription, samples were incubated at 37°C for 1 h.

For PCR, 5 μl of cDNA were heated to 95°C for 5 min and then combined with PCR Reaction Buffer (Fisher Scientific, Pittsburgh, PA); 2.5 mM each of dATP, dCTP, dGTP, and dTTP (Fisher Scientific); 1.25 U of *Taq* DNA Polymerase (Fisher Scientific); 1.5 mM $MgCl_2$ (Fisher Scientific); and 0.5 μM of the appropriate primer pair in MicroAmp reaction tubes (Perkin-Elmer, Norwalk, CT) to achieve a final sample volume of 50 μl. Amplification of samples was performed using the GeneAmp System 9600 thermocycler (Perkin-Elmer). Each sample was denatured at 94°C for 30 s, annealed at 55°C for 30 s, and extended at 72°C for 30 s for 30 cycles. This was followed by a 10-min extension at 72°C. PCR products were analyzed by electrophoresis on a 1.8% agarose gel and stained with ethidium bromide. Size markers (100-bp ladder; Life Technologies) were run adjacent to samples to confirm that PCR products were the expected sizes for each set of primers. Primer pairs used were as follows: HY sense, AAA TGC AGC TCG GAC CAA ATC; HY antisense, CTG AAT GAT GTG AAG CTG TC; β-actin sense, TGG AAT CCT GTG GCA TCC ATG AAA C; and β-actin antisense, TAA AAC GCA GCT CAG TAA CAG TCC G.

HY Restimulation Experiments. Female mice were immunized subcutaneously in the flank with either 50×10^6 syngeneic male splenocytes or 1×10^6 MB49 cells. After 2 weeks, the mice were sacrificed and the spleens removed. After pooling at least two spleens per group, splenocytes were isolated by repeated flushing with PBS followed by passage through a 40 mesh CELLECTOR screen (Thomas Scientific, Swedesboro, NJ). Splenocytes (7×10^6) were restimulated with syngeneic female or male splenocytes (3×10^6) in duplicate wells of a 24-well plate, in a total of 2 ml of complete medium supplemented with 2-mercaptoethanol. Restimulation cultures were carried on for 3 days at 37°C, 5% CO_2, at which point supernatants from duplicate wells were pooled and analyzed for IFN-γ, IL-4, and IL-10 by ELISA, using paired antibodies purchased from PharMingen (San Diego, CA). As a positive control for our IL-4 ELISA, MB49 cells were infected for 2 h with a vaccinia virus-IL-4 construct (made in our laboratory) and then cultured overnight to allow for IL-4 secretion by infected cells. Supernatants from these cultures were assayed for IL-4 on the same plates as experimental supernatants to confirm that the IL-4 assay was functioning properly. The detection limit of our assay is 30 pg/ml.

Determination of IL-10 at Tumor Site *in Vivo*. Female C57BL/6 and female IL-10 KO mice were immunized subcutaneously in the flank with 1×10^6 MB49 cells. After 2 weeks, the mice were sacrificed and the tumors removed. Tumor masses were homogenized into a single cell suspension using a Pyrex tissue grinder followed by passage through a 70 μm nylon cell strainer. Single cell suspensions derived from tumors, as well as MB49 cells grown exclusively *in vitro*, were cultured at 5×10^6 cells/well in a total of 2 ml complete medium in 24-well plates overnight at 37°C, 5% CO_2. Supernatants from duplicate wells were pooled and analyzed for IL-10 by ELISA, using paired antibodies purchased from PharMingen.

Tumor Growth Experiments. Female C57BL/6 mice and female IL-10 KO mice were injected subcutaneously in the flank with 1×10^6 MB49 cells. Cages were given numerical codes to blind the experiment. Tumor size was measured every 2–3 days with metric calipers by measuring the two largest diameters. Mice were sacrificed when one diameter of the tumor became >1.5 cm because at this point mice consistently became sick and lethargic. In our experience, no tumor with a single diameter >0.75 cm has ever been observed to regress. The experiment was unblinded, and Kaplan-Meier survival curves were tested for statistically significant differences, using the Mantel-Haenszel test for comparison of survival curves. Analysis was performed using WINKS Statistical Data Analysis software (TexaSoft, Cedar Hill, TX).

***β*-Gal Immunization.** Female C57BL/6 mice and female IL-10 KO mice were immunized subcutaneously in the flank with 1×10^6 MB49 cells. After 10 days, mice were injected with $1–2 \times 10^6$ pfu recombinant vaccinia virus expressing the *β-galactosidase* gene (vv/β-gal; provided by Dr. Laurence Eisenlohr, Thomas Jefferson University, Philadelphia, PA) in a total of 100 μl directly into the tumor mass or subcutaneously into the flank opposite the tumor. Control mice were injected with $1–2 \times 10^6$ pfu vv/β-gal subcutaneously into the flank. Eight days after the vv/β-gal inoculation, the mice were sacrificed and the spleens removed. After at least two spleens were pooled per group, splenocytes were isolated as described above and restimulated (4×10^6) in quadruplicate wells of a 24-well plate with 20, 10, 5, or 0 μg/ml soluble β-gal (Sigma Chemical, St. Louis, MO) in a total of 2 ml of complete medium supplemented with 2-mercaptoethanol. Restimulation cultures were performed for 3 days at 37°C, 5% CO_2, and supernatants from quadruplicate wells were pooled and analyzed for IFN-γ, IL-4, and IL-10 by ELISA, using paired antibodies purchased from PharMingen. As a positive control for our IL-4 ELISA, supernatants from MB49 cells infected with a vaccinia virus-IL-4 construct were used as described above.

RESULTS

MB49 Expresses the HY Antigen. Summerhayes *et al.* (26) originally described MB49 as a cell line derived by exposure of male C57Bl/6 bladder epithelial cells to the carcinogen dimethylbenz[a]anthracene. Because MB49 was derived from male bladder epithelium, we examined the possibility that MB49 expresses the male transplantation antigen, HY (27). RT-PCR was performed on MB49, using primers that amplify a 250-bp fragment of HY containing the epitope recognized by HY-specific CTL clones (28). As shown in Fig. 1, MB49 expresses HY mRNA when grown *in vitro* (*Lane 3*) and *in vivo* (*Lane 4*). Furthermore, although MB49 tumors grow progressively in both male and female mice, immunization of female mice with syngeneic male spleen cells protects against tumor growth upon subse-

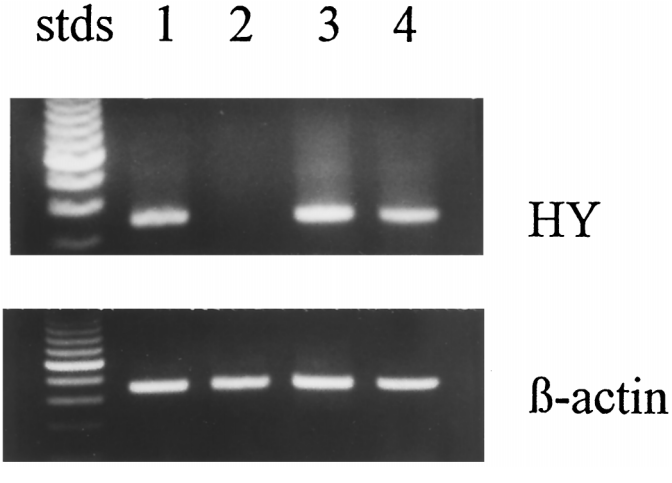

Fig. 1. MB49 expresses the HY antigen. mRNA was extracted from male spleen (*Lane 1*), female spleen (*Lane 2*), MB49 *in vitro* (*Lane 3*), and tumor tissue from MB49 grown in female mice (*Lane 4*). Samples were reverse transcribed and subjected to PCR using primers specific for a 250-bp fragment of HY containing the epitope recognized by HY-specific CTL clones. Primers specific for a 349-bp fragment of β-actin were used for positive controls.

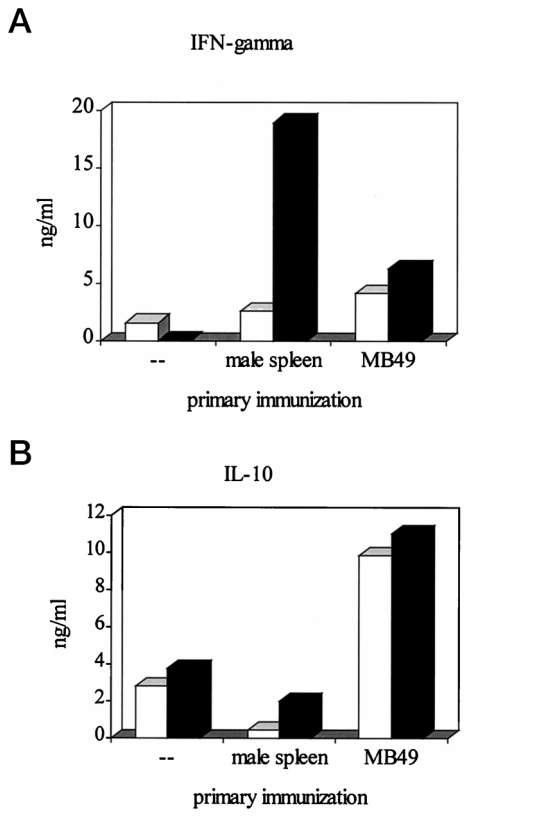

Fig. 2. MB49 fails to prime for an HY-specific type 1 (IFN-γ) response in normal mice. Female C57Bl/6 mice were immunized subcutaneously in the flank with either 50×10^6 syngeneic male spleen cells or 1×10^6 MB49 cells. After 2 weeks, splenocytes were isolated, pooled within experimental groups, and placed into culture with (*black columns*) or without (*open columns*) restimulation by syngeneic male spleen cells. After 3 days, culture supernatants were tested by ELISA for IFN-γ (*A*), IL-10 (*B*), and IL-4. IL-4 was not detectable in experimental supernatants despite our ability to detect IL-4 in control supernatants (see "Materials and Methods") and, therefore, is not shown.

quent challenge with MB49.[4] This indicates that in addition to gene expression, MB49 expresses the HY antigen in a manner that is recognizable by the immune system of syngeneic female mice. The expression of HY by MB49 and its growth in female mice enabled us to use HY as a surrogate tumor antigen in subsequent studies.

MB49 Fails to Prime for an HY-specific Type 1 (IFN-γ) Response in Normal Female Mice. Rejection of HY-expressing male skin grafts by female mice has been shown to be a cell-mediated process requiring both $CD4^+$ and $CD8^+$ T cells (29). In addition, there is mounting evidence that a cell-mediated, type 1 response is most suitable for the elimination of tumor cells (4, 5). Given the link between a type 1 response and the rejection of both HY and of tumor cells, we studied the nature of the cytokine response to HY expressed on MB49 as a measure of antitumor immunity. First, it was necessary to characterize the normal cytokine response to HY. To this end, female C57Bl/6 mice were injected with 50×10^6 syngeneic male spleen cells subcutaneously. After 2 weeks, the mice were sacrificed, the spleens removed, and the splenocytes placed into culture for 3 days with or without restimulation by syngeneic male spleen cells. After 3 days in culture, supernatants were tested by ELISA for the type 1 cytokine, IFN-γ, and the type 2 cytokines, IL-4 and IL-10. Fig. 2 shows that immunization with male spleen cells leads to a male-specific, IFN-γ response (Fig. 2*A*) with very low levels of the type 2 cytokine IL-10 (Fig. 2*B*). IL-4 was not detectable in these cultures, despite our ability to detect IL-4 in positive-control supernatants assayed in parallel (see "Materials and Methods"). Because MB49 expresses the HY antigen, it might be expected to elicit a similar HY-specific, type 1 immune response when injected into female mice. However, as shown in Fig. 2*A*, immunization with MB49 followed by restimulation with male spleen does not elicit a comparable IFN-γ response. In contrast, IL-10 is produced in large quantities by splenocytes of MB49 immunized mice, although this IL-10 production does not appear to be HY-specific (Fig. 2*B*). As will be discussed below, because MB49 induces IL-10 production, we cannot conclude that the presence of IL-10 is an indication of a tumor-specific type 2 response. Rather, failure to detect IL-4 would suggest that no tumor-specific type 2 response is mounted.

IL-10 Is Present at the MB49 Tumor Site in Normal, But Not IL-10 KO Mice. Recently, our group has observed IL-10 production by primary cultures derived from human bladder tumors (30). Given this finding, the presence of IL-10 in the spleens of tumor-bearing mice (see Fig. 2*B*), and the association of IL-10 with a myriad of other types of cancer, we sought to determine whether IL-10 was present at the tumor site in our MB49 system.

IL-10 is not detectable in supernatants of the MB49 cell line when tested by ELISA (Fig. 3). The absence of IL-10 production *in vitro*, however, does not preclude the presence of this cytokine at the site of MB49 tumors *in vivo*. In fact, other groups have reported that tumors are capable of producing soluble factors that induce host cells to produce IL-10 (31, 32). To determine whether the growth of MB49 induces IL-10 production *in vivo*, normal and IL-10 KO mice were injected with 1×10^6 MB49 cells subcutaneously. Two weeks later, tumors were removed, homogenized into single cell suspensions, and put into culture for 18 h. IL-10 was measured in culture supernatants by ELISA. If MB49 is capable of inducing IL-10 production by infiltrating cells, we predicted that only cell suspensions derived from normal mice would secrete IL-10 because infiltrating cells in IL-10 KO mice are genetically incapable of IL-10 production. As shown in Fig. 3, single cell suspensions derived from tumors established in normal mice secrete IL-10, whereas those derived from IL-10 KO mice do not. These data suggest that although MB49 does not produce IL-10 *in vitro* or *in vivo*, it induces infiltrating cells to produce IL-10. The possibility remains that a factor present in normal, but not IL-10 KO, mice induces MB49 to produce IL-10 *in vivo*, and it is with this caveat that we use the phrase "tumor-induced IL-10" throughout the article. However, we view this possibility as highly unlikely and, for

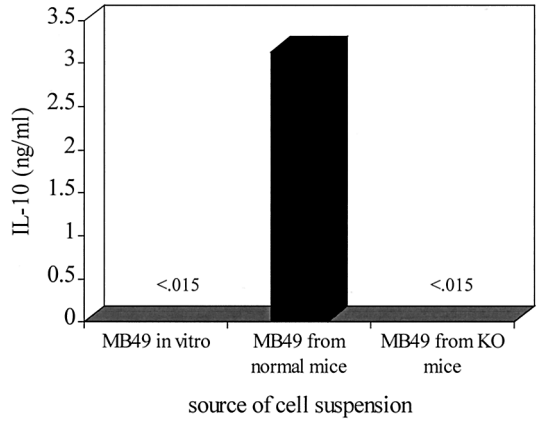

Fig. 3. MB49 induces infiltrating cells to produce IL-10. Female C57BL/6 and female IL-10 KO mice were immunized subcutaneously in the flank with 1×10^6 MB49 cells. After 2 weeks, tumors were removed and homogenized into single cell suspensions. Single cell suspensions derived from tumor, as well as MB49 cells grown exclusively *in vitro*, were put at 5×10^6 cells/well in a total of 2 ml complete medium and cultured overnight. Supernatants from duplicate wells were pooled and analyzed for IL-10 by ELISA.

[4] Unpublished observations.

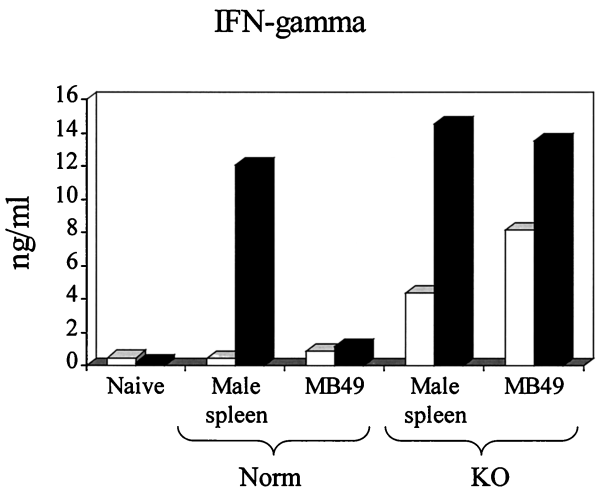

Fig. 4. MB49 does prime for an HY-specific type 1 (IFN-γ) response in IL-10 KO mice. Female C57Bl/6 normal and IL-10 KO mice were immunized subcutaneously in the flank with either 50×10^6 syngeneic male spleen cells or 1×10^6 MB49 cells. After 2 weeks, splenocytes were isolated, pooled within experimental groups, and placed into culture with restimulation by either syngeneic female (*open columns*) or male (*black columns*) spleen cells. After 3 days, culture supernatants were tested by ELISA for cytokines.

the purposes of this study, the absence of IL-10 at the tumor site in the KOs (Fig. 3) renders these animals a suitable model for study of the growth of MB49 in the absence of IL-10. The mechanism of IL-10 induction by MB49 presently is under investigation in our laboratory.

IL-10 KO Mice Are Able To Mount an HY-specific Type 1 (IFN-γ) Immune Response to MB49. To test the hypothesis that the tumor-induced IL-10 is responsible for the lack of an HY-specific IFN-γ response to MB49 (see Fig. 2*A*), we compared the immune response to MB49 in normal *versus* IL-10 KO mice. Female C57Bl/6 normal or IL-10 KO mice were injected subcutaneously with either 50×10^6 syngeneic male spleen cells or 1×10^6 MB49 cells. After 2 weeks, the mice were sacrificed, the spleens removed, and the splenocytes placed into culture for three days with restimulation by either syngeneic female or male spleen cells. After 3 days in culture, the supernatants were removed and tested for both type 1 and type 2 cytokines by ELISA. Consistent with the results shown in Fig. 2, normal mice do not mount an HY-specific IFN-γ response after tumor growth (Fig. 4), but do show nonspecific IL-10 production and no detectable IL-4 (data not shown). In contrast, IL-10 KO mice show a significant HY-specific IFN-γ response (Fig. 4). Increased nonspecific IFN-γ production (*e.g.*, production after restimulation with female spleen) was also observed in IL-10 KO mice, consistent with the elevated spontaneous IFN-γ production seen by others in these animals (33, 34). However, it is important that in six of seven similar experiments, IL-10 KO mice showed male-specific IFN-γ production, whereas normal mice showed a male-specific response in only two of seven similar experiments. These data suggest that the MB49-induced IL-10 is indeed responsible for the absence of a tumor-specific, type 1 immune response in normal mice.

IL-10 KO Mice Show Prolonged Survival and an Increased Frequency of Tumor Rejection. To determine whether the presence of an HY-specific, type 1 response in the above mice altered the growth of MB49 tumors, we compared the long-term survival of tumor-bearing normal *versus* IL-10 KO mice. IL-10 KO mice show significantly prolonged survival ($P = 0.022$, Mantel-Haenszel test for comparison of survival curves) after inoculation with 1×10^6 MB49 cells subcutaneously (Fig. 5). IL-10 KO mice also showed an enhanced ability to reject MB49 tumors when compared with normal mice. In normal mice, only 1 animal of 15 was able to reject the tumor, whereas all other tumors grew progressively to a size that required sacrifice of the animals (Table 1). In contrast, 6 of 15 IL-10 KO mice rejected the tumor completely (after initial tumor take) and remained tumor free for 11 weeks after tumor inoculation (Table 1). To assay for the presence of long-lived antitumor immunity, mice were rechallenged with an equivalent dose of MB49 as the original tumor challenge on the opposite flank. All mice that had rejected tumor previously were resistant to subsequent tumor challenge, whereas three of three naive normal controls developed progressively growing tumors (Table 1). Interestingly, three of three naive IL-10 KO controls grew tumors initially, but two of these three regressed completely (Table 1), consistent with the enhanced ability of IL-10 KO mice to reject primary tumor challenge described above.

MB49-assocated IL-10 Blocks a Type 1 (IFN-γ) Immune Response Directed at a Non-Tumor Antigen Present at the Tumor Site. Although our data demonstrate appropriate HY expression, to ensure that our observations were not somehow unique to this antigen as expressed by the tumor, we examined the immune response to another antigen present at the tumor site. Viral constructs carrying the β-gal gene have been shown previously to elicit a Th1 (IFN-γ) response to β-gal in mice (35). Thus, we used a vaccinia virus carrying the β-gal gene (vv/β-gal) to deliver β-gal to the tumor site and then studied the effect of tumor-induced IL-10 on the generation of an immune response to β-gal.

To confirm that immunization with vv/β-gal would lead to a type 1 response under normal conditions, we injected vv/β-gal subcutaneously into female C57BL/6 mice, waited 8 days, and then restimulated the splenocytes *in vitro* with soluble β-gal. Three-day supernatants

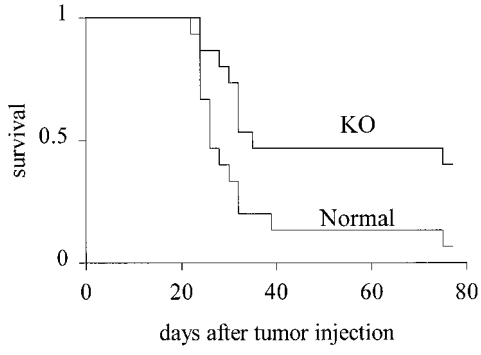

Fig. 5. IL-10 KO mice show prolonged survival after inoculation with MB49. Female C57BL/6 normal mice and IL-10 KO mice were injected subcutaneously in the flank with 1×10^6 MB49 cells. The experiment was blinded, and tumor size was determined every 2–3 days with metric calipers by measuring the two largest diameters. Mice were sacrificed when one diameter of the tumor became >1.5 cm because at this point mice consistently become sick and lethargic and there remained no chance for regression. IL-10 KO mice showed significantly prolonged survival compared with normal mice ($P = 0.022$; Mantel-Haenszel test). Analysis was performed using WINKS Statistical Data Analysis software (TexaSoft). As shown, one normal and one IL-10 KO mouse died at a late time point (75 days after inoculation) due to slow-growing tumors; however, it is important to note that all remaining mice were completely tumor-free at this time point and were thus used in the rechallenge experiment (see Table 1).

Table 1 *Tumor rejection by normal versus IL-10 KO mice*

Normal ($n = 15$) and KO ($n = 15$) mice were challenged with 1×10^6 MB49 cells, and tumor growth was monitored; all mice showed an initial period of tumor growth; those that subsequently rejected tumor and remained tumor-free at 11 weeks were re-challenged with 1×10^6 MB49 cells in the flank opposite initial challenge.

	Initial challenge	Re-challenge
Normal	1/15	1/1
Normal controls[a]		0/3
IL-10 KO	6/15	6/6
IL-10 KO controls[a]		2/3

[a] These mice were used as naive controls for side-by-side comparison with mice re-challenged with MB49 after rejecting initial challenge.

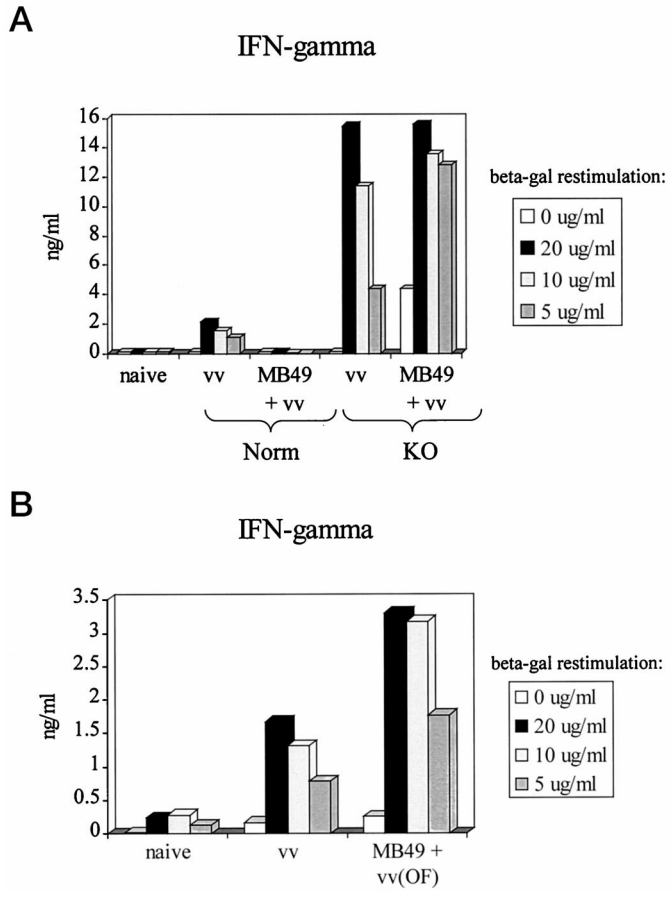

Fig. 6. Tumor-induced IL-10 also blocks the generation of a type 1 (IFN-γ) immune response directed at a non-tumor antigen present at the tumor site. Female C57BL/6 mice and female IL-10 KO mice were immunized subcutaneously in the flank with 1×10^6 MB49 cells. After 10 days, mice were injected with 1×10^6 to 2×10^6 pfu vaccinia-β-gal (vv/β-gal; *vv*) in a total of 100 μl directly into the tumor mass (*A*) or into the opposite flank (*OF*) as the tumor (*B*). Control mice were injected with 1×10^6 to 2×10^6 pfu vv/β-gal subcutaneously into the flank. Eight days after vv/β-gal inoculation, mice were sacrificed and spleens were removed. After at least two spleens were pooled per group, splenocytes were isolated and 4×10^6 splenocytes were restimulated *in vitro* with 20, 10, 5, or 0 μg/ml soluble β-gal. After 3 days, supernatants from quadruplicate wells were pooled and analyzed for cytokines by ELISA.

were tested by ELISA for IFN-γ, IL-10, and IL-4. As shown in Fig. 6*A*, the expected β-gal-specific IFN-γ response was observed. Injection of the same viral construct into a MB49 tumor mass (10 days after inoculation with tumor cells) resulted in no β-gal-specific IFN-γ response. Consistent with prior experiments (see Fig. 2), these MB49-bearing animals showed high levels of non-antigen-specific IL-10 and no detectable IL-4 (data not shown). In contrast, IL-10 KO mice showed a β-gal-specific response after immunization with the vv/β-gal even when immunization was performed directly into the MB49 tumor mass (Fig. 6*A*). These data suggest that the failure of normal, tumor-bearing mice to mount a type 1 response to vv/β-gal present at the tumor site is due to tumor-induced IL-10.

To test whether the presence of tumor-induced IL-10 results in a systemic immune suppression in tumor-bearing animals, vv/β-gal was injected subcutaneously into the opposite flank of the tumor-bearing animal and the response of the splenocytes to *in vitro* restimulation with soluble β-gal was examined. As seen in Fig. 6*B*, tumor-bearing mice mount a β-gal-specific IFN-γ response that is equal to the response mounted by non-tumor-bearing controls, suggesting that the effects of IL-10 are local and do not result in global immune suppression in the tumor-bearing animal.

DISCUSSION

MB49 is a murine transitional cell carcinoma that we have shown to express the male-specific transplantation antigen, HY. Despite its expression of HY, MB49 fails to prime for an HY-specific, type 1 (IFN-γ) immune response. We have shown that *in vivo* growth of MB49 is associated with IL-10 production, most likely by infiltrating host cells. Given this finding, the availability of IL-10 KO mice of the appropriate background, whose infiltrating cells are unable to produce IL-10, allowed us to study the effect of tumor-induced IL-10. In IL-10 KO mice, MB49 primes for an HY-specific, type 1 (IFN-γ) response. Furthermore, IL-10 KO mice show prolonged survival and an increased capacity to reject tumors after inoculation with MB49. These findings demonstrate that tumor-induced IL-10 can block the generation of a type 1 immune response directed against a tumor antigen and thereby can allow for progressive tumor growth. Finally, using a recombinant vaccinia virus vector to deliver a model antigen (β-gal) to the tumor site, we show that the effects of tumor-induced IL-10 are not limited to the tumor antigen, but also suppress the generation of an immune response directed at a non-tumor antigen present at the tumor site.

The profile of cytokines produced during an immune response correlates with and may direct the response toward either cell-mediated immunity, termed type 1, which is marked by delayed-type hypersensitivity and CTLs, or humoral immunity, termed type 2, which is marked by antibody production. As the immune system has become more fully understood, it has become apparent that the *type* of immune response elicited is critical to the ultimate effectiveness of that response. This concept is best illustrated by the example of murine *L. major*. Resistant mouse strains mount a type 1 response (characterized by IFN-γ) to the parasite and effectively resolve the infection, whereas susceptible strains mount a type 2 response (characterized by IL-4 and IL-10) and as a result, die from infection (3). Responses to *Mycobacterium leprae* demonstrate that this same phenomenon exists in humans because patients with the lepromatous form of the disease display a type 2 response (IL-4 and IL-10), whereas patients with the milder tuberculoid form of disease show a type 1 response (IFN-γ; Ref. 36). A more complete list of diseases where the Th1/Th2 dichotomy plays an important role can be found in a recent review by Mosmann and Sad (1).

In tumor systems, a type 1 response is thought to be most effective for the eradication of malignant cells. Aruga *et al.* (4) have demonstrated that $CD3^+$ tumor-draining lymph node cells that secrete a type 1 profile of cytokines can mediate tumor regression in adoptive immunotherapy, whereas those expressing a type 2 cytokine profile are ineffective. In humans, spontaneous regression of melanoma has been correlated with the presence of an elevated type 1 cytokine profile at the tumor site (5). Accordingly, we previously have proposed that one mechanism of immune escape used by tumors is the preferential production or induction of type 2 cytokines at the tumor site (12). The presence of these type 2 cytokines might serve to suppress an effective type 1 antitumor response and/or to promote an ineffective type 2 response.

IL-10 is one such type 2 cytokine, which we and others have shown to be present in a wide variety of human cancers (12–16). Through its effects on APCs, IL-10 can block the development of a type 1 response, thereby favoring the development of a type 2 response (6–11). The lack of tumor-specific IL-4 production in our studies indicates that there may not be development of a type 2 response in our system. Rather, we observe that IL-10 suppresses the generation of a tumor-specific type 1 response without a concomitant increase in a measurable type 2 response. This is consistent with several published reports of IL-10 acting as a strict suppressor of various stages of the immune

response. First, it has been reported that IL-10 can block the accumulation of both macrophages and DCs at the tumor site (37, 38). Because these APCs are thought to be crucial for the initiation of any cell-mediated response, their absence could prevent the initiation of immune responses specific for tumor-associated antigens. Second, DCs that do reach the tumor site might be altered functionally as a consequence of exposure to IL-10. For example, studies have demonstrated that DCs treated with IL-10 show reduced capacity to stimulate T cells and are capable of inducing antigen-specific anergy (39). Finally, tumor-specific T cells that become activated in the presence of IL-10 may differentiate into Tr1 cells, as has been suggested by Groux *et al.* (11). These Tr1 cells have been shown to suppress immune responses both *in vitro* and *in vivo*. Determination of which of these or other mechanisms may be operative in our system is the focus of ongoing studies in our laboratory.

Our findings are consistent with several recent reports that suggest that IL-10 may interfere with antitumor immune responses in other transplantable tumor systems. Qin *et al.* (37) have shown that successful vaccination with granulocyte macrophage colony-stimulating factor gene-modified tumor cells occurs only when the IL-10 normally associated with this tumor is blocked by antisense. Failure to block this IL-10 results in a lack of recruitment of DCs to the tumor site. Donawho *et al.* (40) have demonstrated that the enhanced outgrowth of transplantable melanomas in UV-irradiated mice is due to IL-10. Finally, Hagenbaugh *et al.* (41) have reported that transgenic mice who overproduce IL-10 show accelerated tumor growth that can be slowed by addition of blocking antibody to IL-10. In the present study, we offer the first detailed analysis of the effect of tumor-induced IL-10 on the immune response directed against a tumor antigen. This is the first report in which the removal of tumor-induced IL-10 is itself sufficient to allow for the generation of a tumor-specific type 1 immune response and to allow for complete tumor rejection in up to 40% of animals.

Our data do not appear to be consistent with studies showing that transfection of tumors with the IL-10 gene leads to an increased antitumor immune response and decreased tumorigenicity (22–25). At least two points may explain this apparent conflict. First, it is important to note that the majority of studies correlating IL-10 with decreased tumorigenicity use IL-10-transfected tumors (22–25). Such transfected tumors may not reflect the natural production of IL-10 accurately in terms of concentration, timing, and location. We have circumvented this limitation by using a tumor that does not produce IL-10, but rather induces host cells to produce IL-10. By using this tumor in syngeneic IL-10 KO mice, we were able to study the effects of IL-10 "naturally" associated with the tumor. Our findings show that, in this context, IL-10 can indeed allow the tumor to avoid an immune response that might otherwise be directed against it. An alternative explanation is the possibility that different tumors are inherently different in their response to IL-10. These differences might be related to the presence or absence of other cytokines in the tumor microenvironment, which may influence the ultimate effect of IL-10, or may involve differential susceptibility to different effector mechanisms of the immune system. Therefore, it is conceivable that the differences observed between our system and the transfected-tumor systems may simply reflect inherent differences in the tumor systems studied.

The ability of tumor-induced IL-10 to inhibit the generation of immune responses at the tumor site has important implications with respect to local immunotherapy of cancer. To date, the only widely used form of local immunotherapy is intravesical *BCG* treatment of superficial bladder cancer following surgery. Although *BCG* is relatively effective at initiating a local inflammatory reaction, there is no evidence of tumor-specific cell-mediated immunity following treatment. Consistent with this are our own observations that treatment of intravesical MB49 with *BCG* does not lead to the induction of MB49-specific CTLs.[5] A similar situation exists in melanoma, where only anecdotal evidence exists for the presence of systemic tumor-specific immune responses after localized treatment with *BCG* (42). Our findings suggest that tumor-induced IL-10 may be one reason for the failure of *BCG* to elicit tumor-specific cell-mediated immunity. The presence (or absence) of other cytokines such as IL-2 and granulocyte macrophage colony-stimulating factor, although not examined in the present study, may also contribute to the overall effectiveness of immunotherapeutic treatments. These and other cytokines will be examined as we continue our analysis of the MB49 system.

Certain next-generation strategies of immunotherapy might also be limited by tumor-induced IL-10. Intralesional cytokine gene therapy, presently in clinical trials in our group for melanoma, is one such approach (43, 44). The goal of such therapy is to stimulate the induction of tumor-specific immunity by providing cytokine(s) at the tumor site that are known to promote cell-mediated immunity. The effectiveness of such an approach might be severely limited if IL-10 is also present at the lesion site, potentially blocking or opposing the effects of the delivered cytokine(s). These limitations might be overcome if an effective method of blocking IL-10 is devised and delivered along with the desired cytokine. Fortunately, the effects of tumor-induced IL-10 that we observe in our system are local rather than global (Fig. 6). Therefore, other immunotherapeutic approaches such as vaccination strategies that deliver tumor antigens at sites distant from the primary tumor, and thus away from tumor-induced IL-10, are less likely to be affected.

In summary, our studies lend support to the hypothesis that production/induction of IL-10 by tumor cells may reflect an escape mechanism used by tumor cells to avoid destruction by the immune system. These findings highlight the importance of considering tumor-induced IL-10 in the design of immunotherapeutic strategies aimed at generating tumor-specific cell-mediated immunity and suggest that neutralization of IL-10 may be key to the effectiveness of such therapies.

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